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(57) Abstract

Vascular endothelial growth factor-B (VEGF-B) and vascular endothelial growth factor-C (VEGF-C) are angiogenic polypeptides. It has been shown that VEGF-B and -C are angiogenic *in vitro* especially in combination with bFGF. VEGF-C also increases plasminogen activator (PA) activity in bovine endothelial cell lines and this is accompanied by a concomitant increase in PA inhibitor-1. Addition of alpha-2-antiplasmin to bovine endothelial cells co-treated with bFGF and VEGF-C partially inhibits collagen gel invasion.

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STIMULATION, MODULATION AND/OR INHIBITION OF ENDOTHELIAL PROTEOLYTIC ACTIVITY AND/OR ANGIOGENIC ACTIVITY

Background of the Invention

Angiogenesis is the formation of new capillary blood vessels by a process of sprouting from pre-existing vessels, and occurs during development as well as in a number of physiological and pathological settings. Angiogenesis is thus necessary for tissue growth, wound healing and female reproductive function, and is also a component of pathological processes such as tumor growth, hemangioma formation and ocular neovascularization (see Folkman, *New Engl. J. Med.*, 333:1757-1763 (1995); Pepper, *Arterioscler. Thromb. Vasc. Biol.*, 17:605-619 (1997)). A similar although far less well studied process also occurs in the lymphatic system, and is sometimes referred to as lymphangiogenesis.

Angiogenesis begins with localized breakdown of the basement membrane of the parent vessel, which is followed by the migration and outgrowth of endothelial cells into the surrounding extracellular matrix, resulting in the formation of a capillary sprout. A lumen is subsequently formed, and constitutes an essential element in functional sprout formation. Sprout maturation is completed after reconstitution of the basement membrane. Alterations in at least three endothelial cell functions occur during this series of events: 1) modulation of interactions with the extracellular matrix, which requires alterations in cell-matrix receptors and the production of matrix-degrading proteolytic enzymes (plasminogen activators, PAs, and matrix metalloproteinases, MMPs); 2) an initial increase and subsequent decrease in locomotion migration, which allows the cells to translocate towards the angiogenic stimulus and to stop [illegible] and 3) [illegible]

proliferation, which provides new cells for the growing and elongating vessel, and a subsequent return to the quiescent state once the vessel is formed. Together, these cellular functions contribute to the process of capillary morphogenesis, i.e. the formation of three-dimensional patent or open tube-like structures. Many newly formed capillaries subsequently undergo a process of vessel wall maturation (i.e. formation of a smooth muscle cell-rich media and an adventitia, while others undergo regression (i.e. in the absence of blood flow. [see Pepper et al., Enzyme Protein, 49:138-162 1996.; Risau, Nature 386:671-674 (1997.)].

It is usually stated that with the exception of angiogenesis which occurs in response to tissue injury or in female reproductive organs, endothelial cell turnover in the healthy adult organism is very low. The maintenance of endothelial quiescence is thought to be due to the presence of endogenous negative regulators, since positive regulators are frequently detected in adult tissues in which there is apparently no angiogenesis. The converse is also true, namely that positive and negative regulators often co-exist in tissues in which endothelial cell turnover is increased. This has lead to the notion of the "angiogenic switch", in which endothelial activation status is determined by a balance between positive and negative regulators. In activated (angiogenic) endothelium, positive regulators predominate, whereas endothelial quiescence is achieved and maintained by the dominance of negative regulators [Hanahan et al., Cell, 86:353-364 (1996.)]. Used initially in the context of tumor progression to describe the passage from the prevascular to the vascular phase, the notion of the "switch" can also be applied in the context of developmental, physiological as well as pathological angiogenesis. Although it still remains to be definitively demonstrated in vivo, the current working hypothesis is that the "switch" involves the induction of a positive regulator and/or the loss of a negative regulator.

A number of polypeptide growth factors or cytokines have been

demonstrated to be angiogenic in vivo [see Klagsbrun et al., *Ann. Rev. Physiol.*, 53:217-39 (1991); Leek et al., *J. Leukocyte Biol.*, 56:423-435 (1994); Pepper et al., *Curr. Topics Microbiol. Immunol.*, 213/II:31-67 (1996)]. These include vascular endothelial growth factor (VEGF), also known as vascular permeability factor or vasculotropin, and basic fibroblast growth factor (bFGF). However, although a role for VEGF in the development of the embryonic vasculature and in tumor angiogenesis has been unequivocally established [see Carmeliet et al., *Nature*, 380:435-39 (1996); Ferrara et al., *Nature*, 380:439-442 (1996); Dvorak et al., *Amer. J. Pathol.*, 146:1029-1039 (1995); Ferrara et al., *Endocrine Rev.*, 18:4-25 (1997)], the precise role of bFGF in the endogenous regulation of angiogenesis remains to be established. Nonetheless, two observations point to the potential importance of interactions between these two cytokines in the regulation of angiogenesis. First, VEGF and bFGF have been demonstrated to synergize in the induction of angiogenesis in vitro [Pepper et al., *Biochem. Biophys. Res. Commun.*, 189:824-831 (1992); Goto et al., *Lab. Invest.* 69:508-517 (1993)], and this observation been confirmed in vivo in the rabbit in a model of hind-limb ischemia [Asahara et al., *Circulation*, 92(Supp.II):II.365-71 (1995)] and in the rat in a sponge implant model [Hu et al., *A. Br. J. Pharmacol.*, 114:262-268 (1995)]. Second, the in vitro angiogenic effect of VEGF as well as its capacity to induce PA activity are both dependent on endogenous bFGF produced by endothelial cells.

Alterations in endothelial cell function induced by members of the VEGF family are mediated via transmembrane tyrosine kinase receptors which at present include VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) [see Mustonen et al., *J. Cell Biol.*, 129:895-898 (1995)]. VEGFRs are expressed in many adult tissues, despite the apparent lack of constitutive angiogenesis. VEGFRs are however clearly up-regulated in endothelial cells during development and in certain angiogenesis-associated/dependent pathological situations including tumor growth [see Dvorak et al.,

Amer. J. Pathol., 146:1029-1039 (1995); Ferrara et al., Endocrine Rev., 18:4-25 (1997)]. The phenotypes of VEGFR-1-deficient mice and VEGFR-2-deficient mice reveal an essential role for these receptors in blood vessel formation during development. VEGFR-1-deficient mice die in utero at mid-gestation due to inefficient assembly of endothelial cells into blood vessels, resulting in the formation of abnormal vascular channels [Fong et al., Nature, 376:66-70 (1995)]. VEGFR-2-deficient mice die in utero between 8.5 and 9.5 days post-coitum, and in contrast to VEGFR-1, this appears to be due to abortive development of endothelial cell precursors [Shalaby et al., Nature 376:62-66 (1995)]. The importance of VEGFR-2 in tumor angiogenesis has also been clearly demonstrated by using a dominant-negative approach [Millauer et al., Nature, 367:576-579 (1994); Millauer et al., Cancer Res. 56:1615-1620 (1996)]. The phenotype of VEGFR-3-deficient mice has not yet been reported.

The ligands for VEGFR-1 include VEGF and placenta growth factor (PlGF); ligands for VEGFR-2 include VEGF and VEGF-C; while the only ligand reported so far for VEGFR-3 is VEGF-C [see Mustonen et al., *J. Cell Biol.*, 129:895-898 (1995); Thomas, *J. Biol. Chem.*, 271:603-606 (1996)]. VEGF-C is a recently described member of the VEGF family of angiogenic cytokines. It is a protein with structural homology to VEGF, which was isolated from the human prostatic adenocarcinoma cell line PC-3 during a search for a ligand for VEGFR-3 [see Joukov et al., *EMBO J.*, 15:290-298 (1996)]. In an independent search for a ligand for VEGFR-3, VEGF-related protein (VRP) was isolated from a human G61 glioma cell cDNA library screened with probes based on an expressed sequence tag ("EST") encoding an amino acid sequence with a high degree of similarity to VEGF [Lee et al., Proc. Natl. Acad. Sci. USA 93:1988-92 (1996)]. VEGF-C and VRP are the same protein, and will be referred to as VEGF-C from here on. VEGF-C displays a high degree of similarity with VEGF, including conservation of the eight cysteine residues involved in intra- and intermolecular disulfide bonding. The cysteine-rich C-terminal half, which

increases the length of the VEGF-C VHF polypeptide relative to other ligands of this family, shows a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein repeat. The C-terminal propeptide also contains short motifs of VEGF-like domains, which may promote the interaction of secreted VEGF-C with the extracellular matrix. VEGF-C binds to the extracellular domain of VEGFR-3 and induces VEGFR-3 tyrosine phosphorylation. In addition to VEGFR-3, VEGF-C binds to and induces phosphorylation of VEGFR-2. VEGF-C promotes the growth of human and bovine endothelial cells, although it is less active than VEGF in this assay. VEGF-C has been reported to induce endothelial cell migration in three-dimensional collagen gels [Joukov et al., *EMBO J.*, 16:3898-911 (1997)]. VEGF-C transcripts are detectable in many adult and fetal human tissues and in a number of cell lines. Human VEGF-C has been mapped to chromosome 4q34 [Paavonen et al., *Circulation* 93:1079-82 (1996)].

One of the striking features of VEGF-C is that its mRNA is first translated into a precursor from which the mature ligand is derived by cell-associated proteolytic processing. Following biosynthesis, VEGF-C rapidly associates into a 58 kDa antiparallel homodimer linked both by disulfide and non-covalent bonds. This is followed by proteolytic processing of both N- and C-terminal propeptides in the terminal portion of the secretory pathway and at the cell membrane, giving rise to a number of incompletely processed intermediates. Mature VEGF-C is then released from cells as a 21 kDa homodimer containing two VEGF-homology domains linked by non-covalent interactions. As a consequence of this processing, VEGF-C acquires the ability to bind to and activate VEGFR-2, and also increases its affinity for and activating properties towards VEGFR-3. Intracellular proteolytic cleavage is not a prerequisite for VEGF-C secretion. Based on these observations, it has been suggested that the synthesis of VEGF-C as a precursor allows it to diffuse relatively through the [illegible] [Joukov et al., EMBO J., 16:3898-911 (1997)], which is restricted to venous endothelium during early stages of development and which

becomes restricted to lymphatic endothelium later in development and in postnatal life [Kukk et al., Development, 122:3829-3837 (1996)]. Under certain circumstances, in which the processing mechanism becomes activated, it has been suggested that VEGF-C may acquire the additional capacity to signal through VEGFR-2, thereby providing an additional level of regulation of VEGF-C activity. Signalling through VEGFRs requires receptor dimerization. Proteolytic processing might provide a further regulatory mechanism by indirectly promoting the formation of VEGFR-2/VEGFR-3 heterodimers.

Using an in vitro model of angiogenesis which assays for extracellular matrix invasion and tube formation as described by Montesano et al., *Cell* 42:469-477 (1985), it has been reported that bFGF and VEGF induce bovine microvascular, lymphatic and aortic endothelial cells grown on three-dimensional collagen or fibrin gels to invade the underlying matrix within which they form capillary-like tubular structures [see Montesano et al., *Proc. Natl. Acad. Sci. USA*, 83:7297-7301 (1986); Pepper et al., *Exp. Cell Res.*, 210:298-305 (1994); Pepper et al., *J. Cell Sci.*, 108:73-78 (1995)]. This demonstrates that the angiogenesis-inducing properties of bFGF and VEGF can be mediated via a direct effect on endothelial cells. However, there is increasing evidence that the nature of the response elicited by a specific cytokine is contextual, i.e., depends on the presence or absence of other regulatory molecules in the pericellular environment of the responding cell. With respect to angiogenesis, it has previously been demonstrated that VEGF and bFGF synergize in an in vitro model [Pepper et al., *Biochem. Biophys. Res. Commun.*, 189:824-831 (1992)], and that in the same model, TGF-β1 has a biphasic effect [Pepper et al., *Exp. Cell Res.*, 204:356-363 (1993)].

Despite intensive activity in the art, there has remained a need for ways to influence angiogenesis. In particular there is a need for methods for enhancing, modulating and/or inhibiting angiogenesis, and for methods for stimulating or inhibiting the

activity of proteolytic enzymes, such as plasminogen activators.

Summary of the Invention

It is an object of the present invention to characterize VEGF receptor expression in bovine endothelial cell lines;

Another object of the invention is to develop a method of synergistically inducing the angiogenic response of endothelial cells.

A further object of the invention is to provide a method of influencing the proteolytic properties of endothelial cells.

It is also an object of the invention to provide a method of suppressing an angiogenic response in endothelial cells.

Yet another object of the invention is to provide a method of inhibiting the activity of proteolytic enzymes.

It is a specific object of the invention to provide a method of inhibiting the activity of plasminogen activators.

A still further object of the invention is to provide a method of inhibiting tumor growth.

It has now been found that vascular endothelial growth factor-B (VEGF-B) and vascular endothelial growth factor-C (VEGF-C) stimulate endothelial extracellular proteolytic activity, particularly the activity of plasminogen activators, and also synergize with basic fibroblast growth factor (bFGF) to induce angiogenesis in endothelial cells, and that it is possible by administration or co-administration of VEGF-B and/or VEGF-C, as well as other growth factors, to modulate the angiogenic activity of endothelial cells.

In particular, it has now been found that VEGF-C induces angiogenesis in endothelial cells in vitro and that both VEGF-B and VEGF-C synergistically promote angiogenesis in endothelial cells when co-administered with bFGF or VEGF.

The synergistic angiogenesis inducing effect of VEGF-B or VEGF-C with bFGF may be applied in regulation of physiological and pathological angiogenesis, as well as in the planning of therapeutic strategies against tumors and other pathological

conditions.

The fact that the bovine cell lines used in the following tests show a clear response to the human VEGF, VEGF-B and VEGF-C tested indicates that the bovine receptors are sufficiently homologous to the human receptors to give rise to a high degree of inter-species cross reactivity. In view of this, a similar or greater response certainly can be expected in human tissues which express the human receptors.

VEGF-B and VEGF-C induce gene expression of various proteolytic enzymes such as plasminogen activators (PAs), which may damage surrounding tissues and thereby facilitate invasion of those tissues by tumor cells. Also, since VEGF-C can act as a chemoattractant for endothelial cells, this could lead to invasion and permeation by lymphatic vessels (which are composed of endothelial cells) and ultimately support the onset of tumor metastases. Consequently, VEGF-B and VEGF-C antagonists could be used therapeutically to inhibit the action of VEGF-B and/or VEGF-C and thereby retard or prevent permeation, endothelial cell invasion and/or metastasis. In particular, as shown by Example 14 below, α2-antiplasmin can counteract the angiogenic action of VEGF-B and/or VEGF-C and could therefore be used in accordance with one aspect of the invention as an anti-metastatic agent.

Another aspect of the invention involves providing an antisense nucleotide sequence which is complementary to at least a part of the DNA sequence for VEGF-B and/or VEGF-C, which promote proliferation of endothelial cells. The invention thus embraces antisense oligonucleotides that selectively bind to a nucleic acid molecule encoding VEGF-B and/or VEGF-C protein, and their use to decrease transcription and/or translation of VEGF-B or VEGF-C genes, respectively. This is desirable in virtually any medical condition wherein a reduction in VEGF-B and/or VEGF-C gene product expression is desirable, including any reduction in any aspect of a tumor cell phenotype attributable to VEGF-B and/or VEGF-C gene expression. Antisense molecules can be used in this manner to retard or arrest those aspects of a tumor cell phenotype.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, or a modified oligodeoxyribonucleotide, which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and which thereby inhibits transcription of that gene and/or translation of that mRNA. Antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with that gene. Persons skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e. to hybridize substantially more to the target sequence than to another sequence in the target cell under physiological conditions. Based upon the published DNA sequences of VEGF-B and VEGF-C, one of skill in the art can readily select and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 7, and more preferably at least 15, consecutive bases which are complementary to the target [see Wagner et al., *Nature Biotechnology*, 14:840-844 (1996)]. Most preferably, the antisense oligonucleotides comprise a complementary sequence of from 20 to 30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to the N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 5' untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art, but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense

sequence preferably is targeted to sites in which mRNA secondary structure is not expected (see Sainio et al., Cell Mol. Neurobiol., 14:5 (439-457 (1994)) and at which proteins are not expected to bind.

It is considered within the skill of the art to provide either antisense nucleotides which are complementary to a cDNA of the desired gene or antisense nucleotides which hybridize with genomic DNA of the desired gene, since one of ordinary skill in the art may readily derive the genomic DNA corresponding to a cDNA or vice versa. In like manner, antisense to allelic or homologous DNAs may be obtained without undue experimentation.

Antisense oligonucleotides used in the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors. In preferred embodiments, however, antisense oligonucleotides used in the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target, but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e. a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates,

carbamates, carbonates, phosphate triesters, acetamidates, peptides, and carboxymethyl esters. The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. Modified oligonucleotides also can include base analogs such as C-5 propyne modified bases [see Wagner et al., *Nature Biotechnology*, 14:844 (1996)]. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridize under physiological conditions with nucleic acids encoding VEGF-B and/or VEGF-C proteins, together with pharmaceutically acceptable carriers.

Antisense oligonucleotides may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the antisense oligonucleotides in combination with any standard physiologically and/or pharmaceutically acceptable carriers known in the art. The compositions should be sterile and contain a therapeutically effective amount of the antisense oligonucleotides in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

A vector comprising such an anti-sense sequence may be used to inhibit, or at least mitigate expression of the relevant cytokine. The use of a vector of this type to inhibit expression of one or more angiogenic cytokines may be advantageous in instances where expression of the cytokine is associated with a disease such as in instances where tumors produce VEGF-B and/or VEGF-C in order to provide for angiogenesis. Transformation of such tumor cells with a vector containing an anti-sense nucleotide sequence would suppress or retard the endothelial cell proliferating activity and could thereby inhibit permeation, endothelial invasion and/or metastasis and consequently inhibit or retard tumor growth.

Brief Description of the Drawings

Figures 1a through 1f are microphotographs showing angiogenic cell cord invasion of collagen gels induced by VEGF, VEGF-C, bFGF and combinations of VEGF or VEGF-C and bFGF.

Figures 2a and 2b are graphs showing in vitro angiogenesis in bovine microvascular endothelial (BME) cells induced by VEGF-C alone or in combination with bFGF.

Figures 3a and 3b are graphs showing in vitro angiogenesis in BME cells induced by VEGF-B alone or in combination with bFGF.

Figures 4a through 4c are graphs showing in vitro angiogenesis in bovine aortic endothelial (BAE) cells induced by VEGF-C alone or in combination with various concentrations of bFGF.

Figures 5a and 5b are graphs showing in vitro angiogenesis in BAE cells induced by VEGF-B alone or in combination with bFGF.

Figure 6 is a graph showing in vitro angiogenesis induced by VEGF-C in bovine lymphatic endothelial (LEC) cells.

Figure 7 is a graph showing the effects of VEGF and VEGF-C individually and in combination with each other on BAE cell collagen invasion.

Figures 8a and 8b are graphs which also show the effects of VEGF, VEGF-B and VEGF-C individually and in various combinations

in BAE cell collagen invasion.

Figures 9a and 9b are zymographic analyses of the induction of tissue-type plasminogen activator (tPA) activity and urokinase-type plasminogen activator (uPA) activity, and Figure 9c is a reverse zymographic analysis of the induction of plasminogen activator inhibitor-1 (PAI-1) by VEGF-C, VEGF and bFGF.

Figure 10a is a zymographic analysis of the induction of tPA activity and uPA activity, and Figure 10b is a reverse zymographic analysis of the induction of PAI-1 activity, by VEGF-B, VEGF and bFGF.

Figure 11 shows the increase in steady state levels of tPA, uPA and PAI-1 mRNA induced by VEGF-C in BAE cells.

Figure 12 shows the increase in steady state levels of uPA and PAI-1 mRNA induced by VEGF-B in BAE cells.

Figure 13 is a graph showing the inhibiting effect of α_2-antiplasmin on endothelial cell invasion of collagen gels induced by VEGF-C and bFGF in BME cells.

Detailed Description

Recombinant human VEGF-B_{167} and VEGF-B_{186} can be produced as described in Eriksson et al., WO 96/26736, the disclosure of which is incorporated herein by reference.

Recombinant human VEGF-C was produced in the baculovirus system using the Sf9 insect cell line (National Public Health Institute, Helsinki) and baculovirus shuttle vectors derived from the transfer plasmid pFASTBAC1 as described in Joukov V., *Functional Analysis of VEGF-B and VEGF-C*, Helsinki University (1997).

Recombinant human VEGF-C (NNF) was produced in the yeast Pichia pastoris strain X33 using the pPIC9 expression vector (Invitrogen) as described by Joukov et al., EMBO J. 15:290-298 (1996).

Recombinant human VEGF (165-amino acid homodimeric species - VEGF$_{165}$) was purchased from Peprotech.

Recombinant human bFGF (155 amino acid form) was provided by

Dr. A. Barmientos (Farmitalia Carlo Erba, Milan, Italy).

Bovine uPA, bovine uPAR and human tPA cDNA clones were provided by Dr. W.-D. Schleuning.

Example 1: Cell Culture of Microvascular Endothelial Cells.

Bovine adrenal cortex-derived microvascular endothelial (BME) cells (Furie et al., 1984) were obtained from Drs. M.B. Furie and S.C Silverstein and were grown in minimal essential medium, alpha modification (Gibco AG, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (DCS) (Flow Laboratories, Baar, Switzerland), penicillin (500 U/ml) and streptomycin (100ug/ml). Cells were used between passages 16 and 19.

Example 2: Cell Culture of Aortic Endothelial Cells.

Bovine aortic endothelial (BAE) cells, isolated from scrapings of adult bovine thoracic aortas and cloned by limiting dilution as previously described in Pepper et al., *Am. J. Physiol.* 262:C1246-57 (1992), were cultured in low glucose Dulbecco's modified minimal essential medium (DMEM, Gibco) supplemented with 10% DCS and antibiotics. Cells were used between passages 10 and 15.

Example 3: Cell Culture of Lymphatic Endothelial Cells.

Bovine lymphatic endothelial (BLE) cells were isolated from mesenteric lymphatic vessels and provided by Dr. S. Wasi. Passage 3 cells were cloned by limiting dilution as described in Pepper et al., *Exp. Cell Res.*, 210:298-305 (1994). BLE cells were cultured in DMEM supplemented with 1 mM sodium pyruvate, 10% donor calf serum and antibiotics. Cells were used between passages 21 and 26.

Example 4: Cell Culture of Pulmonary Artery Endothelial Cells.

Calf pulmonary artery endothelial (CPAE) cells were purchased from American Type Culture Collection (Rockville, MD), and were cultured in medium 199 supplemented with 20% DCS. Cells were used

at passage 23.

All endothelial cell lines were maintained in 1.5% gelatin-coated tissue culture flasks (Falcon Labware, Becton-Dickinson Company, Lincoln Park, NJ) and subcultured at a split ratio of 1/3 or 1/4. The endothelial nature of all four cell lines has previously been confirmed by DiI-Ac-LDL (Paesel and Lorei, Frankfurt, Germany) uptake and immunostaining with a rabbit polyclonal antiserum against human von Willebrand factor (Nordic Immunology, Tilburg, The Netherlands).

Example 5: Stimulation of Angiogenesis in BME Cells by VEGF or VEGF-C Alone or in Combination with bFGF.

To measure angiogenesis induction in vitro, three-dimensional collagen gels were prepared as described by Montesano et al., Cell 42:469-477 (1985). Eight volumes of a solution of type I collagen from rat tail tendons (approximately 1.6 mg/ml) were quickly mixed with 1 volume of 10x minimal essential medium (Gibco) and 1 volume of sodium bicarbonate (11.76 mg/ml) on ice, dispensed into 18 mm tissue culture wells (Nunclon, A/S Nunc, Roskilde, Denmark), and allowed to gel at 37°C for 10 minutes. Bovine microvascular endothelial (BME) cells were then seeded onto the surface of the collagen gels at 0.5-1.0 x 10^5 cells/well in 500 µl medium. Cells were grown to confluence (3-5 days), at which point serum was reduced to 2% and the cell monolayers were treated with VEGF at a concentration of 30 ng/ml or VEGF-C at a concentration of 0, 1, 3, 10, 30 and 100 ng/ml, alone and in combination with 10 ng/ml of bovine fibroblast growth factor (bFGF). Medium and treatments were renewed every 2 to 3 days, and the cultures fixed and photographed after four days.

Randomly selected fields measuring 1.0 mm x 1.4 mm were photographed in each well at a single level beneath the outer monolayer by phase contrast microscopy at a magnification of 14X using a Nikon Diaphot TMD inverted photomicroscope. Figure 1A is a phase contrast view of an untreated control. Figure 1B shows a sample treated with 30 ng/ml of VEGF. The resultant formation

of cell cords within the collagen gel is clearly apparent, the plane of focus being beneath the surface of the gel. Figure 1c shows a collagen gel treated with 30 ng/ml VEGF-C. Figure 1d shows a sample treated 10 ng/ml bFGF alone. Again a definite invasive response is visible. Figure 1e shows a gel treated with a combination of 30 ng/ml VEGF and 10 ng/ml bFGF, and Figure 1f shows a gel treated with the combination of 30 ng/ml VEGF-C and 10 ng/ml bFGF. The synergistic effects achieved by co-administration of VEGF and bFGF or of VEGF-C and bFGF are readily seen.

The cell cord invasion was quantified by determining the total additive length of all cellular structures which had penetrated beneath the surface monolayer either as apparently single cells or in the form of cell cords as described in Pepper et al., *Biochem. Biophys. Res. Commun.*, 189:824-831 (1992). Results shown graphically in Figures 2a and 2b are expressed as the mean total additive sprout length in $\mu m \pm$ s.e.m., and are from at least nine photographic fields, i.e. three fields from each of at least three separate experiments per condition. $^{a}p < 0.000$, $^{b}p < 0.000$ (Student's unpaired t-test). Mean values were compared using Student's unpaired t-test, and a significant p value was taken as < 0.05. For comparison purposes the results obtained with 30 ng/ml VEGF alone and 30 ng/ml VEGF in combination with 10 ng/ml bFGF are shown by the bar labelled (V-A) at the right side of each graph.

Example 6: Stimulation of Angiogenesis in BME Cells by VEGF-B in Combination with bFGF.

The procedure of Example 5 was repeated, except that BME cell monolayers were treated with VEGF-B_{167} at concentrations of 1, 10 and 100 ng/ml and VEGF-B_{186} at concentrations of 1, 10 and 100 ng/ml alone and in combination with 1 ng/ml bFGF. The shorter 167 amino acid form of VEGF-B lacks the hydrophobic region and the O-glycosylation site seen in the longer 186 amino acid form. The results are illustrated graphically in Figure 3. Although VEGF-B

alone did not produce measurable angiogenesis in this test, VEGF-B_{186} at a concentration of 100 ng/ml and VEGF-B_{167} at all concentrations tested produced a more than additive angiogenesis effect in combination with 10 ng/ml bFGF. For comparison purposes the effects of administration of VEGF and VEGF-C alone and in combination with 10 ng/ml bFGF are shown by the bars labelled V-A and V-C at the right of each graph, respectively.

Example 7: Stimulation of Angiogenesis in BAE Cells by VEGF-C Alone and in Combination with bFGF.

The procedure of Example 5 was again repeated except that the surface of the gel in each well was seeded with a culture of bovine aortic endothelial (BAE) cells, and VEGF-C was tested both alone and in combination with bFGF at concentrations of 1 ng/ml and 10 ng/ml. The results are shown graphically in Figures 4(a), 4(b) and 4(c), respectively. It can be seen that not only does VEGF-C alone stimulate angiogenesis in BAE cells, but a clearly more than additive angiogenesis stimulating effect is observed when cell cultures are treated with VEGF-C in combination with bFGF. Again, the results from comparable treatment with VEGF are shown by the bar labelled (V-A) at the right side of each graph.

Example 8: Stimulation of Angiogenesis in BAE Cells by VEGF-B in Combination with bFGF.

The procedure of Example 6 was repeated except that the surface of the gel in each well was seeded with a culture of bovine aortic endothelial (BAE) cells and bFGF was used at a concentration of 3 ng/ml. The results are depicted graphically in Figures 5(a) and 5(b). Again VEGF-B alone did not produce an apparent angiogenic effect in this test, but both VEGF-B_{186} and VEGF-B_{167} produced a more than additive (i.e. synergistic) angiogenic effect when co-administered with bFGF. For comparison purposes, results obtained with VEGF (V-A) and VEGF-C (V-C) alone and in combination with bFGF are shown at the right side of each graph.

Example 8: Stimulation of Angiogenesis in BLE Cells by VEGF-C.

Bovine lymphatic endothelial (BLE) cell monolayers formed by seeding three-dimensional gels as described in Example 5 with BLE cell cultures were treated with VEGF-C and/or VEGF. After 4 days treatment, randomly selected fields of the gels were photographed at a single level beneath the surface monolayer as described above. Endothelial cell invasion was quantified as described in conjunction with the preceding examples. The results are shown graphically in Figure 6. It can be seen that VEGF-C produced a definite stimulation of angiogenic activity.

The foregoing Examples 5 through 8 demonstrate that the angiogenesis-inducing properties of VEGF-B and VEGF-C can be mediated via a direct effect on endothelial cells and that there is a potent synergistic effect between VEGF-B or VEGF-C and bFGF in the induction of angiogenesis.

As shown by Figures 1c and 2a, when added to confluent monolayers of BME cells on three-dimensional collagen gels, the invasive response induced by VEGF-C was barely measurable. In contrast, Figures 4a and 6 show that VEGF-C induced a definite dose dependent invasive response in BAE and BLE cells, respectively, which was accompanied by the formation of branching tube-like structures as seen by phase-contrast microscopy by focussing beneath the surface monolayer. Assuming a M_r of 42,000 for VEGF-C, when compared at equimolar (0.6-0.7 nM) concentrations, VEGF-C (30 ng/ml) was slightly less potent than VEGF (30 ng/ml), which in turn was about half as potent as bFGF (10 ng/ml) (cf. Figures 4 (a) & (b), 6 and 7).

When co-administered with bFGF, VEGF-C induced a synergistic in vitro angiogenic response. Thus, when added to BME cells, in which VEGF-C alone has little or no effect, VEGF-C potentiated the effects of bFGF, with a maximal 2.5-fold increase at 3.0 ng/ml VEGF-C as shown in Figures 7 (a) & (b). Similarly, in both BAE and BLE cells, in which VEGF-C alone induced angiogenic activity, co-administration of VEGF-C and bFGF induced a greater-than-additive response as shown, for example, in Figure 4.

Example 10: Co-Administration of VEGF and VEGF-C.

The effects of co-addition of VEGF and VEGF-C also were assessed. ($^{b}p < 0.005$, $^{a}p < 0.001$ Student's unpaired t-test). In BME cells, in which only VEGF induces invasion, the response measured for up to 14 days occurred as for VEGF alone. In BLE cells, in which VEGF and VEGF-C each individually induce invasion, the response to co-administration of 30 ng/ml VEGF and 30 ng/ml VEGF-C was essentially additive (see Figure 6). With BAE cells, in which both VEGF and VEGF-C individually induce invasion, the response (measured for to 4 days was far greater than additive as can be seen from the graph of Figure 7.

A three-way comparison of the effects of VEGF, VEGF-B and VEGF-C individually and in combination is shown in the graphs of Figures 8a and 8b. Figure 8a shows the individual effects of the three cytokines. It can be seen that 30 ng/ml of VEGF-B alone produced a negligible effect. VEGF (30 ng/ml) alone produced a marked effect, but the greatest individual effect was produced by 30 ng/ml of VEGF-C. As can be seen from Figure 8b, when VEGF and VEGF-B were co-administered, the result was clearly greater than the sum of their individual effects. Likewise, co-administration of VEGF-B with VEGF-C clearly potentiated the collagen gel invasion inducing activity of VEGF-C. The most striking effect, however, was produced by the combination of 30 ng/ml VEGF and 30 ng/ml VEGF-C.

Example 11: Stimulation of PA and PAI-1 Activity by VEGF-C in BME and BAE cells.

Cell extracts and culture supernatants prepared from BME and BAE cells exposed to VEGF-C at concentrations 0, 1 3 10 30 and 100 ng/ml for 18 hours, were subjected to zymography and reverse zymography. For comparison purposes, test media from BME and BAE cells exposed to 30 ng/ml VEGF and 10 ng/ml bFGF, respectively, were also analyzed. Confluent monolayers in individual wells of 35 mm gelatin-coated tissue culture dishes were washed twice with serum-free medium, and the respective cytokines were added in

serum-free medium containing Trasylol 200 KIU ml . After 15 hours, cell extracts were prepared and analyzed by zymography and reverse zymography as described in Vassalli et al., J. Exp. Med. 159:1653-68 1984 and Pepper et al., J. Cell Biol., 111:743-755 1990 .

Figure 9a shows a zymographic analysis of cell extracts from the BME cells. Figure 9b shows the same zymography incubated for a longer time period at 37°C. Figure 9c shows a reverse zymographic analysis of culture supernatant from BAE cells.

Example 10: Stimulation of PA and PAI-1 Activity by VEGF-B in BME and BAE Cells.

The procedure of Example 11 was repeated except that the culture supernatants were taken from cells exposed to VEGF-B at concentrations of 0, 1, 3, 10, 30 and 100 ng/ml. The results are shown in Figures 10a and 10b. For comparison purposes, the results of exposures to 30 ng/ml VEGF and 10 ng/ml bFGF are also shown.

The zymography and reverse zymography results shown in Figures 9a through 9c indicate that VEGF-C induces uPA, tPA and PAI-1 activity in BME and BAE cells. Similarly, the zymography and reverse zymography results shown in Figures 10a and 10b demonstrate that VEGF-B induces uPA and PAI-1 activity in endothelial cells. The induction of uPA and PAI-1 activity was less than that seen with approximately equimolar concentrations of VEGF. The zymography test results also confirm that bFGF has little or no effect on the expression of tPA. The effect of VEGF-C on PAI-1 activity in BME cells, on uPA activity in BAE cells and on tPA and PAI-1 activity in BLE cells was also tested [illegible] than that shown in Figure 9, [illegible].

Example 11: RNA preparation, in vitro transcription and Northern blot hybridization.

Cytokines were added to confluent endothelial cell monolayers

to which fresh complete medium had been added 24 hours previously. Total cellular RNA was prepared after the indicated times using Trisol reagent (Gibco BRL, Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. Replicate filters containing total cellular RNA (6 μg/lane) prepared from confluent monolayers of BME cells exposed to VEGF-C or VEGF-B (300 ng/ml) for time periods of 0, 1, 3, 9, 24 and 48 hours, were hybridized with ^{32}P-labelled cRNA probes. Controls are shown for time periods of 0, 9, 24 and 48 hours. RNA integrity and uniformity of loading were determined by staining the filters with methylene blue after transfer and cross-linking as shown by the 28S and 18S ribosomal RNAs at the bottom of the Figures 11 and 12. Northern blots, UV cross-linking and methylene blue staining of filters, in vitro transcription, hybridization and post-hybridization washes were as previously described by Pepper et al., *J. Cell Biol.*, 111:743-755 (1990). [^{32}P]-labelled cRNA probes were prepared from bovine urokinase-type plasminogen activator (u-PA) [Kraetzschmar et al., *Gene*, 125:177-83 (1993)], bovine u-PA receptor (u-PAR) [Kraetzschmar et al., *Gene*, 125:177-83 (1993)], human tissue-type PA (t-PA) [Fisher et al., *J. Biol. Chem.*, 260:11223-11230 (1985)] and bovine PA inhibitor-1 (PAI-1) [Pepper et al., *J. Cell Biol.*, 111:743-755 (1990)] cDNAs as described in Pepper et al., *J. Cell Biol.*, 111:743-755 (1990) and Pepper et al., *J. Cell Biol.*, 122:673-684 (1993). It was found that VEGF-C increases steady-state levels of uPA, tPA and PAI-1 mRNA in BAE cells.

The Northern blot analysis shown in Figure 11 indicates that VEGF-C increases steady state levels of uPA, uPAR, tPA and PAI-1 mRNAs in BAE cells. The kinetics of PA, uPAR and PAI-1 induction in were rapid within 1 hour, and transient baseline levels were attained between [illegible] for uPA and uPAR, and between 9 and 24 hours for PAI-1. With respect to uPA and uPAR, this is in contrast to the more sustained increase reported with VEGF and bFGF (see Pepper et al., *J. Cell Biol.*, 111:743-755 (1990); Mandriota et al., *J. Biol. Chem.*, 270:9709-16 (1995)). With respect to PAI-1, the results are similar to VEGF and bFGF. The

Northern blot test results for the tests with VEGF-B are shown in Figure 12.

This co induction of extracellular proteolytic activity, which is manifest by increased synthesis of uPA, and of protease inhibition, which is manifest by increased synthesis of PAI-1, is one of the hallmarks of angiogenesis both in vivo and in vitro and is consistent with the theory of "proteolytic balance", which recognizes that while proteases are necessary for cell migration and morphogenesis, protease inhibitors play an equally important permissive role by protecting the extracellular matrix from inappropriate destruction.

Example 16: Inhibition of Endothelial Cell Angiogenesis in the Presence of VEGF-C and bFGF by α_2-antiplasmin.

BME cell monolayers were co-treated for 4 days with bFGF and VEGF-C as well as α_2-antiplasmin at concentrations of 0, 1, 3, 10 and 30 µg/ml. Randomly selected fields of the treated gels were then photographed at a single level beneath the surface monolayer. Endothelial cell invasion was quantified as described above in Example 5. Figure 13 shows the results in graph form. A clear decrease in angiogenic activity can be seen as the concentration of α_2-antiplasmin increases. It is thus apparent that α_2-antiplasmin inhibits the induction of angiogenesis by co-treatment with VEGF-C and bFGF in a dose-dependent manner.

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed to include everything within the scope of the appended claims and equivalents thereof.

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Vassalli, J.-D., Hamilton, J. and Reich, E. (1976) Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. Cell 8: 271-281.

Watanabe, Y. and Dvorak, H.F. (1997) Vascular permeability factor/vascular endothelial growth factor inhibits anchorage-disruption-induced apoptosis in microvascular endothelial cells by inducing scaffold formation. Exp. Cell Res. 233: 340-349.

CLAIMS

1. A method of stimulating angiogenesis in endothelial cells comprising co-administering to said cells at least two cytokines selected from the group consisting of VEGF, VEGF-B, VEGF-C and FGF.

2. A method according to claim 1, wherein said endothelial cells are selected from the group consisting of microvascular endothelial cells, aortic endothelial cells and lymphatic endothelial cells.

3. A method according to claim 2, wherein said endothelial cells are bovine endothelial cells.

4. A method according to claim 2, wherein said endothelial cells are human endothelial cells.

5. A method according to claim 1, wherein said cells are treated in in vitro culture in a culture medium and the cytokines are administered by incorporating them into the culture medium.

6. A method according to claim 1, wherein the cytokines are administered in the form of purified proteins.

7. A method according to claim 1, wherein the cytokines are administered by transfecting or transforming endothelial cells with at least one vector comprising nucleotide sequences encoding the cytokines operably linked to at least one promoter sequence.

8. A method according to claim 1, wherein the at least two cytokines comprise VEGF-C and FGF.

9. A method according to claim 1, wherein the at least two cytokines comprise VEGF-B and FGF.

10. A method according to claim 1, wherein the at least two cytokines comprise VEGF and VEGF-C.

11. A method according to claim 1, wherein the at least two cytokines comprise VEGF-B and VEGF-C.

12. A method according to claim 1, wherein the at least two cytokines comprise VEGF and VEGF-B.

13. A method of modulating endothelial cell angiogenesis by cells which release a synergistic combination of at least two angiogenic cytokines selected from the group consisting of VEGF, VEGF-B, VEGF-C and FGF, said method comprising neutralizing one of said at least two angiogenic cytokines.

14. A method according to claim 13, wherein said neutralizing is effected by treating the cells with an antibody for the angiogenic cytokine to be neutralized.

15. A method according to claim 14, wherein the cytokine to be neutralized is VEGF-C.

16. A method according to claim 14, wherein the cytokine to be neutralized is VEGF-B.

17. A method according to claim 14, wherein said antibody is a monoclonal antibody.

18. A method according to claim 13, wherein said cells are solid tumor cells.

19. A method of modulating angiogenesis by endothelial cells in the presence of VEGF-C and FGF, said method comprising treating said cells with antiglaucoman.

21. A method according to claim 19, wherein said antiplasmin comprises α_2-antiplasmin.

22. A method according to claim 19, wherein said endothelial cells are selected from the group consisting of microvascular endothelial cells, aortic endothelial cells and lymphatic endothelial cells.

23. A method of stimulating proteolytic activity of endothelial cells comprising the step of treating the cells with VEGF-B, VEGF-C or a combination of VEGF-B or VEGF-C with at least one further cytokine selected from VEGF and FGF.

24. A method according to claim 23, wherein said endothelial cells are selected from the group consisting of microvascular endothelial cells, aortic endothelial cells and lymphatic endothelial cells.

25. A method of inhibiting endothelial cell permeation, invasion and/or metastasis in a patient comprising administering to said patient an effective endothelial cell proliferation inhibiting amount of a VEGF-B or VEGF-C antagonist.

26. A method of modulating angiogenic activity of endothelial cells comprising transfecting or transforming the cells with a vector containing an anti-sense nucleic acid for VEGF-B or VEGF-C.

AMENDED CLAIMS

[received by the International Bureau on 23 December 1998 (23.12.98), original claims 1 and 13 amended, remaining claims unchanged (2 pages)]

1. A method of stimulating angiogenesis in endothelial cells comprising co-administering to said cells a synergistic combination of at least two cytokines selected from the group consisting of VEGF, VEGF-B, VEGF-C and FGF, wherein at least one of said at least two cytokines is VEGF-B or VEGF-C.

2. A method according to claim 1, wherein said endothelial cells are selected from the group consisting of microvascular endothelial cells, aortic endothelial cells and lymphatic endothelial cells.

3. A method according to claim 2, wherein said endothelial cells are bovine endothelial cells.

4. A method according to claim 2, wherein said endothelial cells are human endothelial cells.

5. A method according to claim 1, wherein said cells are treated in in vitro culture in a culture medium and the cytokines are administered by incorporating them into the culture medium.

6. A method according to claim 1, wherein the cytokines are administered in the form of purified proteins.

7. A method according to claim 1, wherein the cytokines are administered by transfecting or transforming endothelial cells with at least one vector comprising nucleotide sequences encoding the cytokines operably linked to at least one promoter sequence.

8. A method according to claim 1, wherein the at least two cytokines comprise VEGF-C and FGF.

9. A method according to claim 1, wherein the at least two cytokines comprise VEGF-B and FGF.

11. A method according to claim 1, wherein the at least two cytokines comprise VEGF and VEGF-C.

11. A method according to claim 1, wherein the at least two cytokines comprise VEGF-B and VEGF-C.

12. A method according to claim 1, wherein the at least two cytokines comprise VEGF and VEGF-B.

13. A method of modulating endothelial cell angiogenesis by cells which release a synergistic combination of at least two angiogenic cytokines selected from the group consisting of VEGF, VEGF-B, VEGF-C and FGF, wherein at least one of said at least two cytokines is VEGF-B or VEGF-C, said method comprising neutralizing one of said at least two angiogenic cytokines.

14. A method according to claim 13, wherein said neutralizing is effected by treating the cells with an antibody for the angiogenic cytokine to be neutralized.

15. A method according to claim 14, wherein the cytokine to be neutralized is VEGF-C.

16. A method according to claim 14, wherein the cytokine to be neutralized is VEGF-B.

17. A method according to claim 14, wherein said antibody is a monoclonal antibody.

18. A method according to claim 14, wherein said cells are solid tumor cells.

19. A method of inhibiting angiogenesis by endothelial cells in the presence of VEGF-C and FGF, said method comprising treating said cells with antiplasmin.

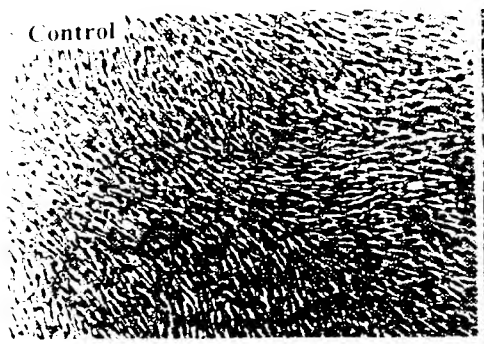

FIG. 1a

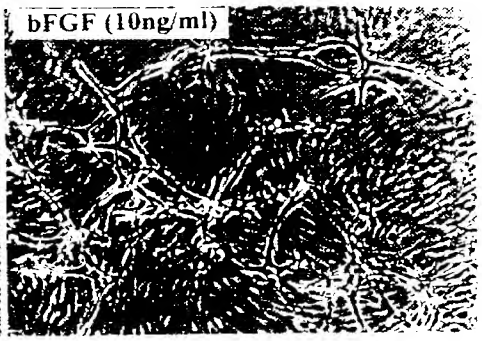

FIG. 1d

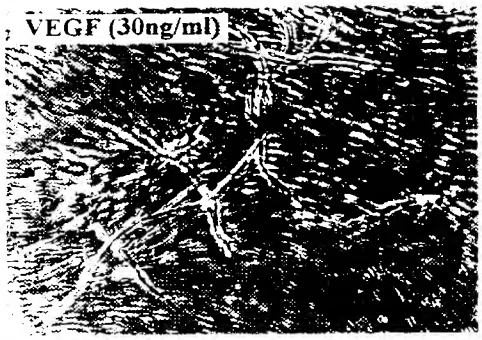

FIG. 1b

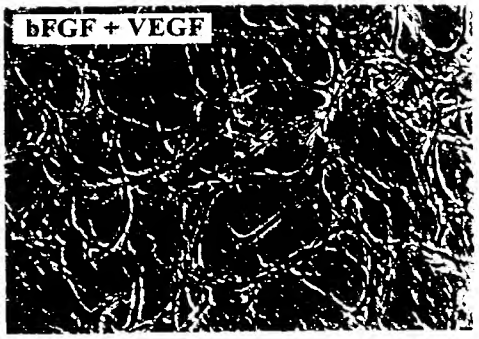

FIG. 1e

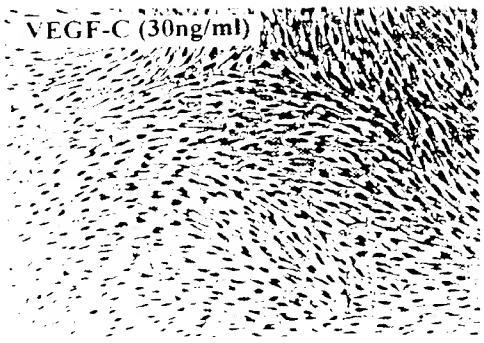

FIG. 1c

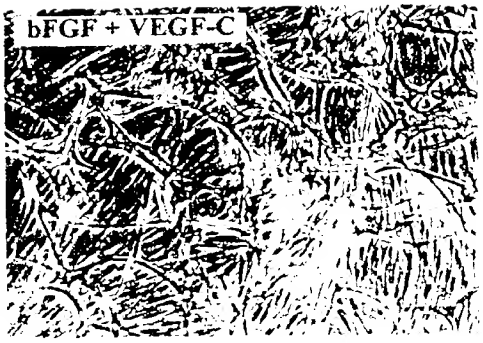

FIG. 1f

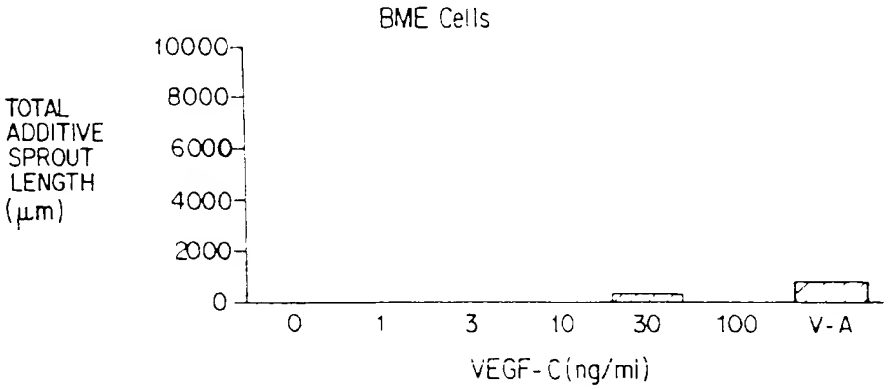
BME Cells
TOTAL ADDITIVE SPROUT LENGTH (μm)
10000
8000
6000
4000
2000
0
0
1
3
10
30
100
V-A
VEGF-C(ng/ml)

FIG. 2a

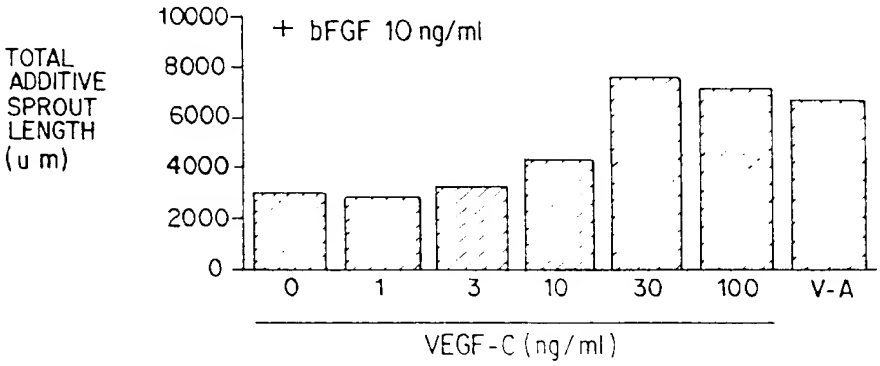
TOTAL ADDITIVE SPROUT LENGTH (u m)
+ bFGF 10 ng/ml
10000
8000
6000
4000
2000
0
0
1
3
10
30
100
V-A
VEGF-C (ng/ml)

FIG. 2b

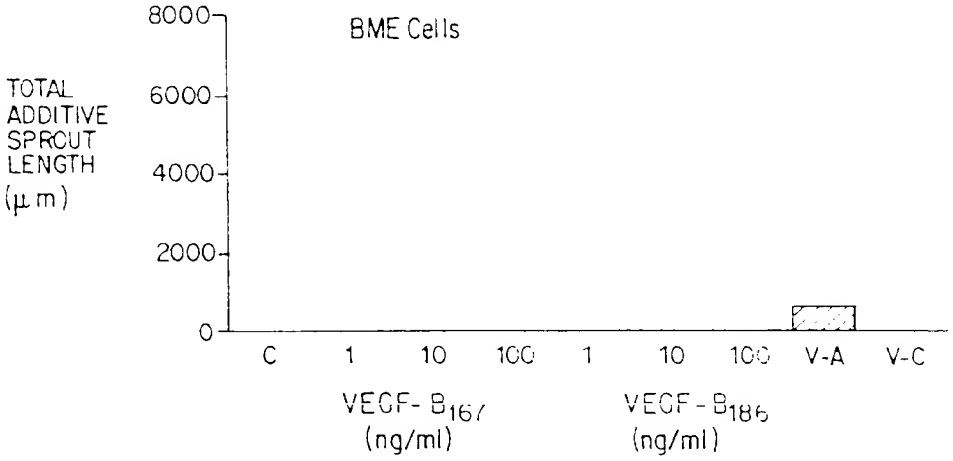
BME Cells
TOTAL ADDITIVE SPROUT LENGTH (μm)
8000
6000
4000
2000
0
C
1
10
100
1
10
100
V-A
V-C
VEGF-B167 (ng/ml)
VEGF-B186 (ng/ml)

FIG. 3a

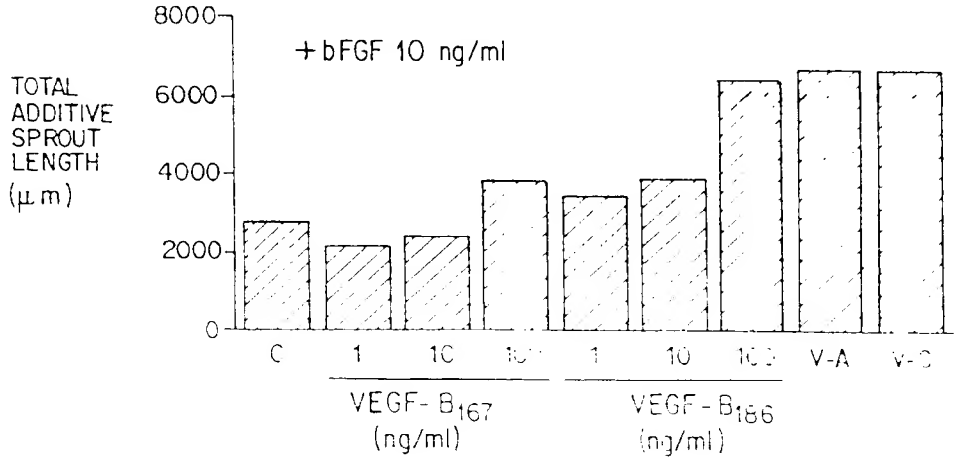
+bFGF 10 ng/ml
TOTAL ADDITIVE SPROUT LENGTH (μm)
8000
6000
4000
2000
0
C
1
10
1
10
V-A
VEGF-B167 (ng/ml)
VEGF-B186 (ng/ml)

FIG. 3b

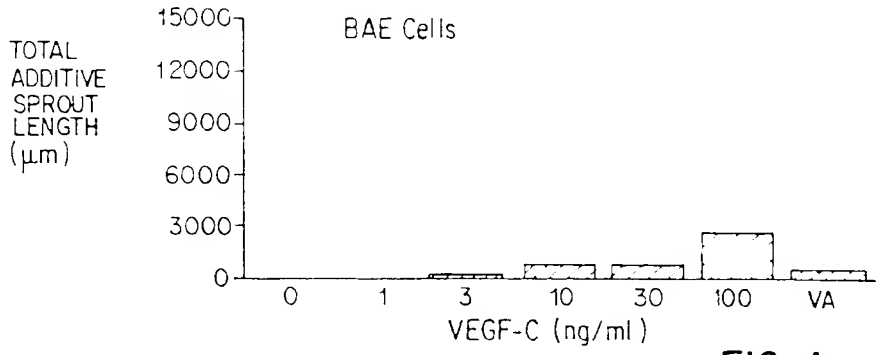

FIG. 4a

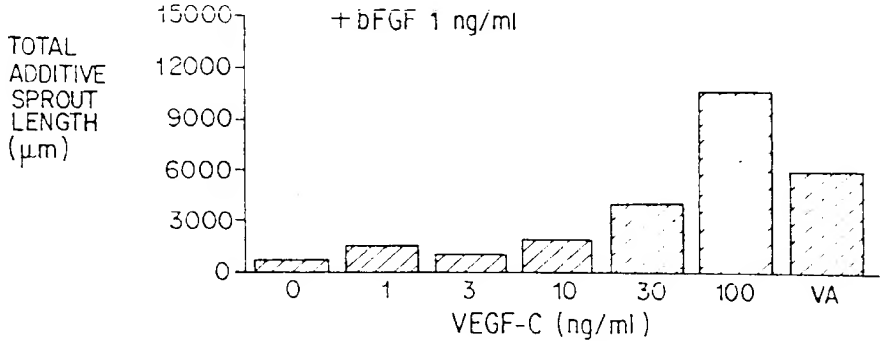

FIG. 4b

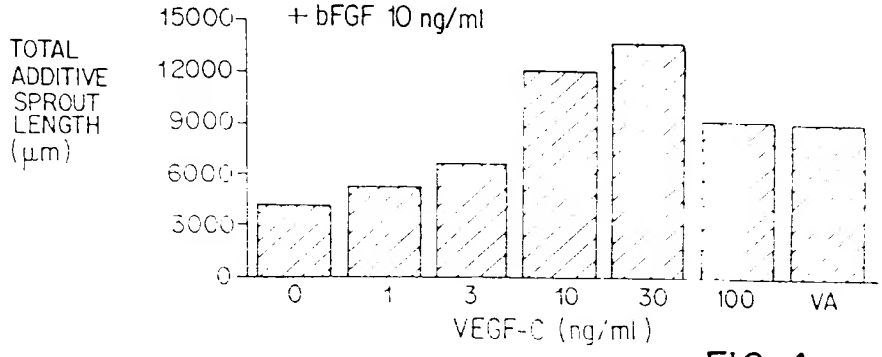

FIG. 4c

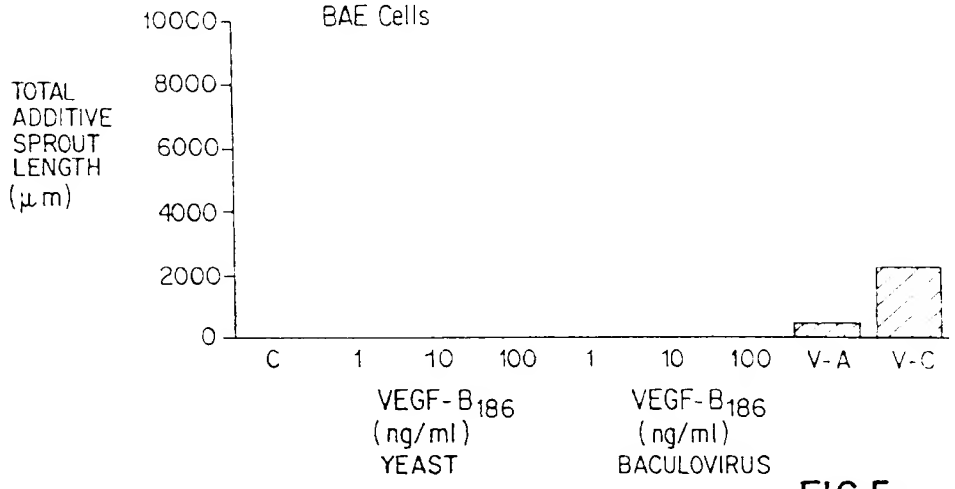
BAE Cells
TOTAL ADDITIVE SPROUT LENGTH (μm)
10000
8000
6000
4000
2000
0
C
1
10
100
1
10
100
V-A
V-C
VEGF-B186 (ng/ml) YEAST
VEGF-B186 (ng/ml) BACULOVIRUS

FIG.5a

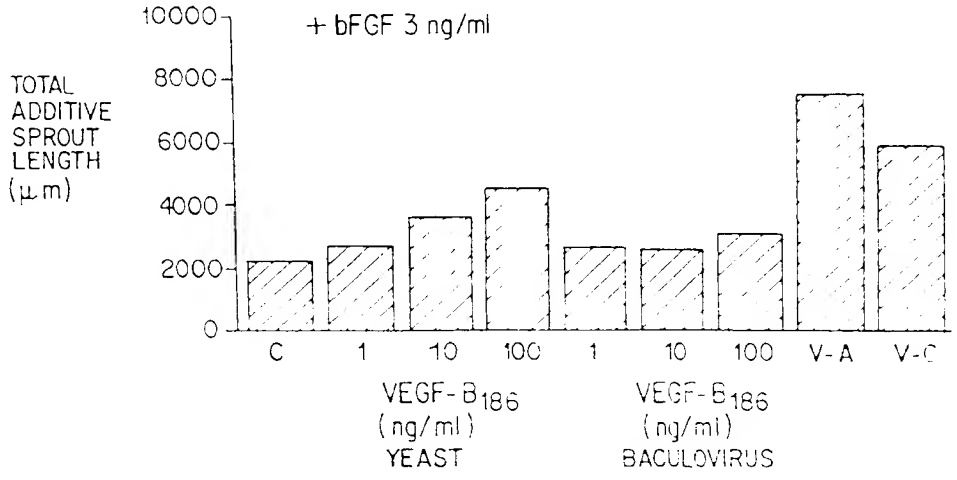
+ bFGF 3 ng/ml
TOTAL ADDITIVE SPROUT LENGTH (μm)
10000
8000
6000
4000
2000
0
C
1
10
100
1
10
100
V-A
V-C
VEGF-B186 (ng/ml) YEAST
VEGF-B186 (ng/ml) BACULOVIRUS

FIG.5b

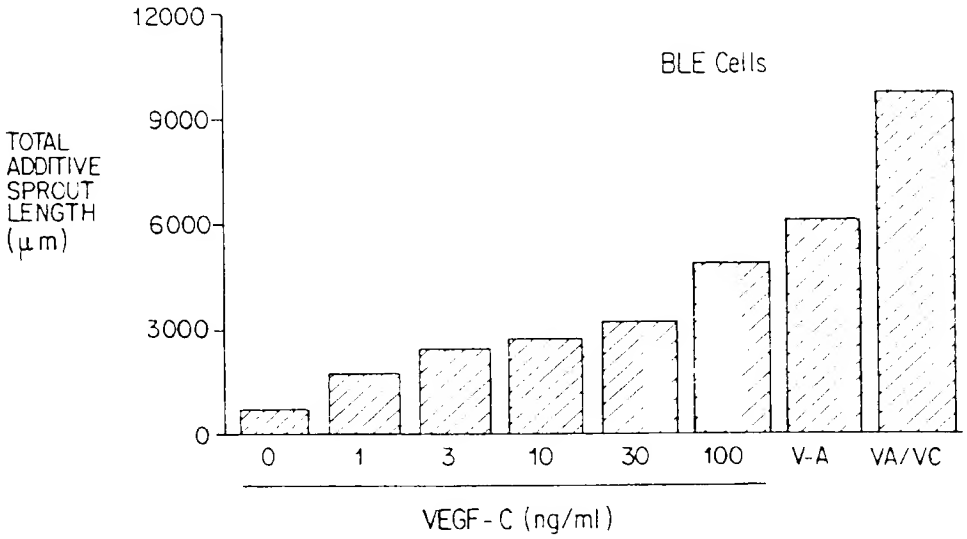
12000
9000
6000
3000
0
BLE Cells
TOTAL ADDITIVE SPROUT LENGTH (μm)
0
1
3
10
30
100
V-A
VA/VC
VEGF-C (ng/ml)

FIG. 6

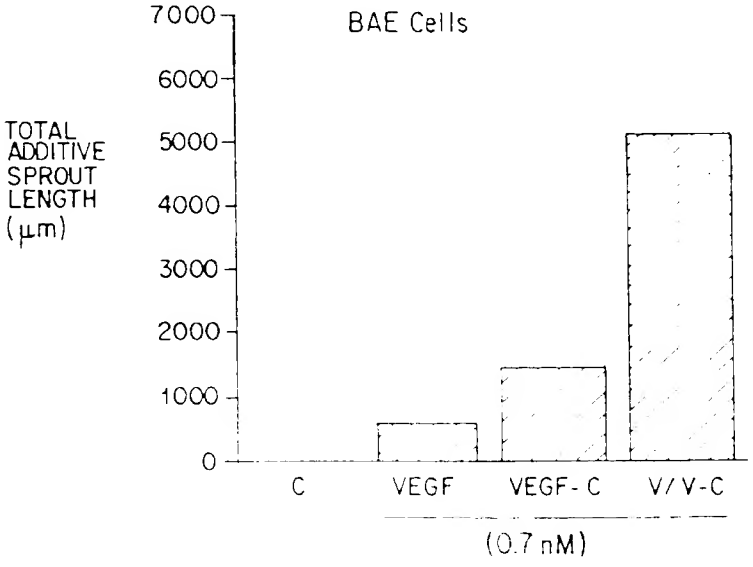
7000
6000
5000
4000
3000
2000
1000
0
BAE Cells
TOTAL ADDITIVE SPROUT LENGTH (μm)
C
VEGF
VEGF-C
V/V-C
(0.7 nM)

FIG. 7

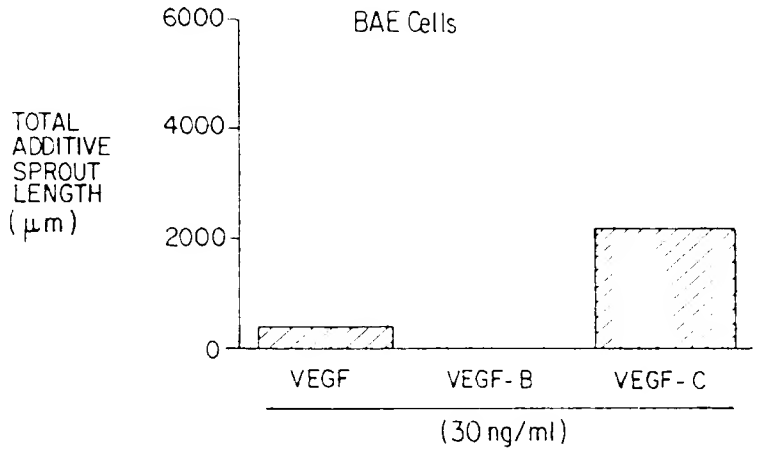
BAE Cells
TOTAL ADDITIVE SPROUT LENGTH (μm)
6000
4000
2000
0
VEGF
VEGF-B
VEGF-C
(30 ng/ml)

FIG. 8a

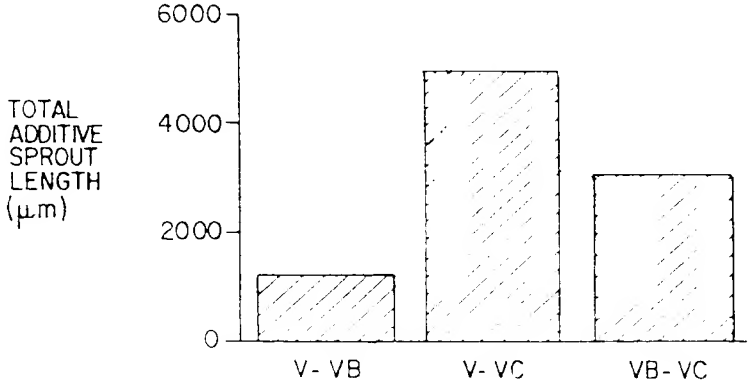
TOTAL ADDITIVE SPROUT LENGTH (μm)
6000
4000
2000
0
V-VB
V-VC
VB-VC

FIG. 8b

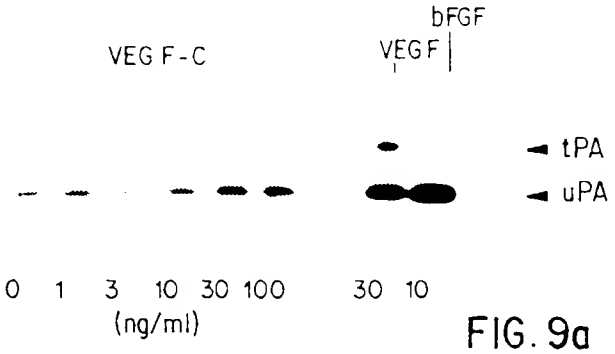

FIG. 9a

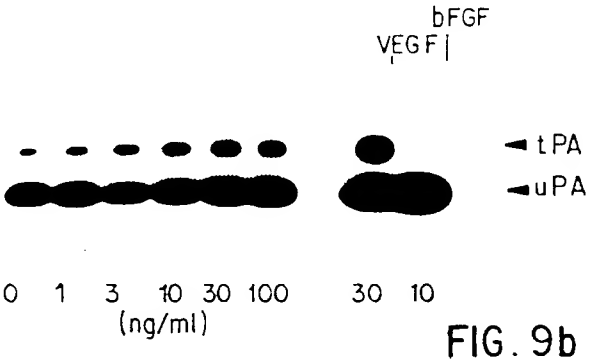

FIG. 9b

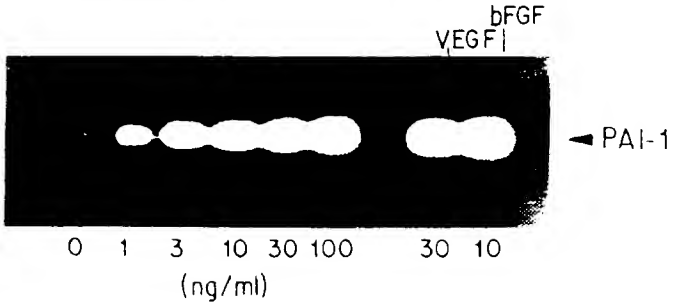

FIG. 9c

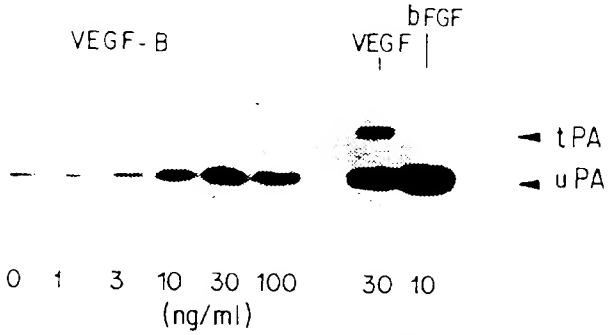

FIG. 10a

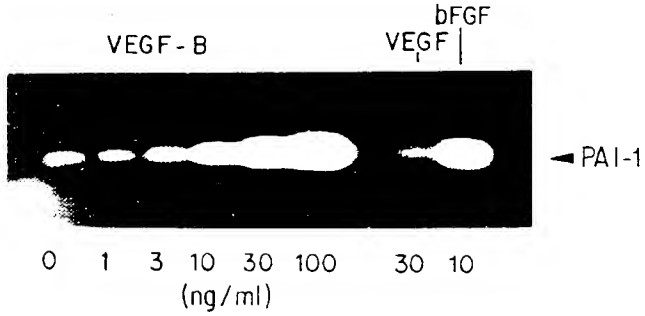

FIG. 10b

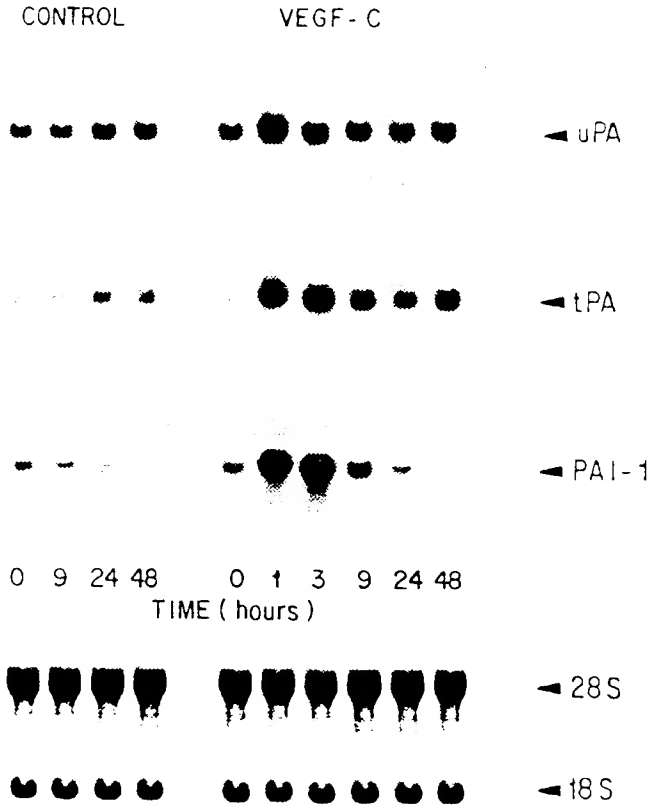
CONTROL
VEGF-C
uPA
tPA
PAI-1
0 9 24 48 0 1 3 9 24 48
TIME (hours)
28S
18S

FIG. 11

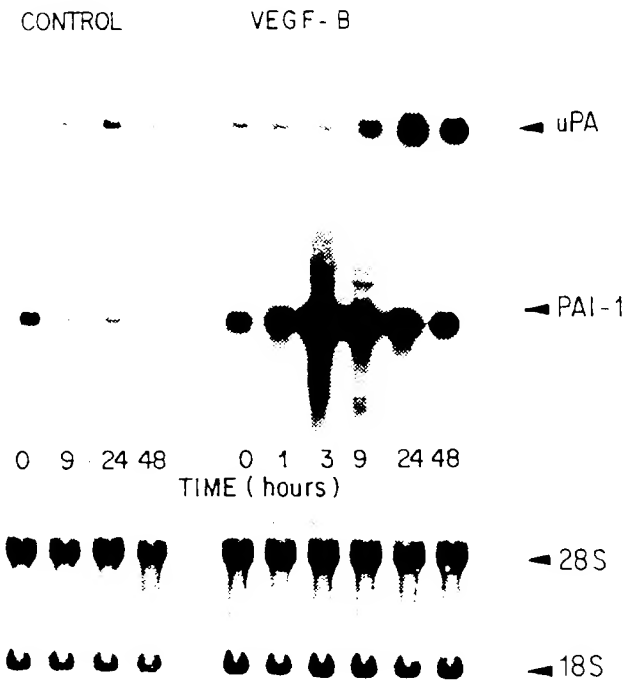

FIG. 12

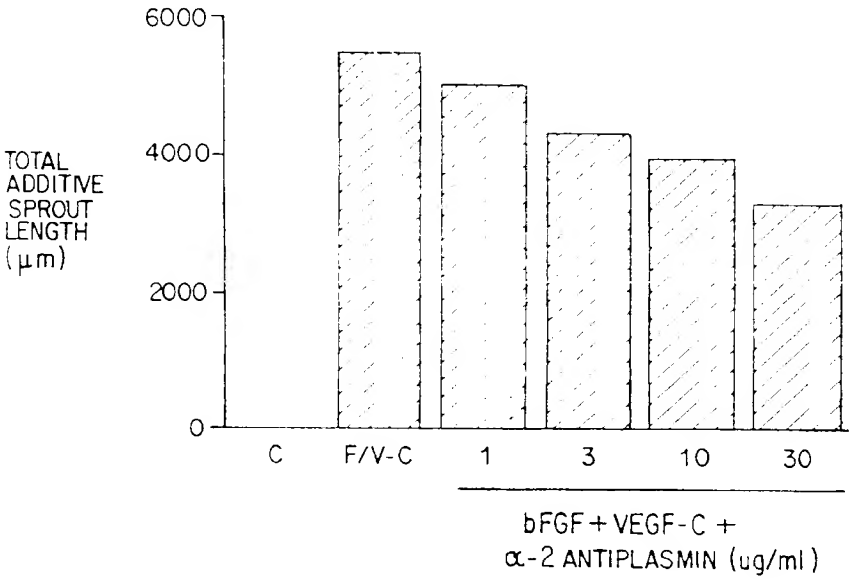
6000
4000
2000
0
TOTAL ADDITIVE SPROUT LENGTH (μm)
C
F/V-C
1
3
10
30
bFGF + VEGF-C +
α-2 ANTIPLASMIN (ug/ml)

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16816

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) Please See Extra Sheet.
US CL 435/6, 172.3, 373, 404, 405; 514/2, 44; 536/24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 172.3, 373, 404, 405; 514/2, 44; 536/24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BATTEGAY, E.J. ET AL. Angiogenesis: Mechanistic insights, neovascular diseases, and therapeutic prospects. Journal of Molecular Medicine. 1995, Vol. 73, No. 7, pages 333-346, see entire document.	1-25
Y	ENHOLM, B. ET AL. Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. Oncogene. 1997, Vol. 14, pages 2475-2483, see entire document.	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 OCTOBER 1998	29 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer
BRIAN R. STANTON

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16816

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HU, G-F. ET AL. Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities. Proceedings of the National Academy of Sciences, USA. December 1994, Vol. 91, pages 12096-12100, see entire document.	1-25
Y	PEPPER, M.S. ET AL. Leukemia inhibitory factor (LIF) inhibits angiogenesis in vitro. Journal of Cell Science. 1995, Vol. 108, pages 73-83, see entire document.	1-25
Y	KOOLWIJK, P. ET AL. Cooperative effect of TNF-alpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of Urokinase activity. The Journal of Cell biology. March 1996, Vol. 132, No. 6, pages 1177-1188, see entire document.	1-25
Y	PEPPER, M.S. ET AL. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochemical and Biophysical Research Communications. 15 December 1992, Vol. 189, No. 2, pages 824-831, see entire document.	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16816

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 37/18, 43/04; C7H 21/04; C12N 5/00, 5/09, 5/10, 15/09, 15/12; C12Q 1/68

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, Medline, Biosis, CA
Search Terms:vegf?; vascular; endothel?; growth; factor?; angiogen?; microvasc?; fgf; bfgf; basic; fibroblast?; antibod?, inhibit?; antiplasmin; anti; sense; protein?; pepper?/au; alitalo?/au; eriksson?/au